Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

2-phenylpropene; α-methylstyrene

EC Number: 202-705-0
CAS Number: 98-83-9

CLH-O-0000007252-80-01/F

Adopted

16 March 2023

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16 March 2023

CLH-O-0000007252-80-01/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

Chemical name: **2-phenylpropene; α-methylstyrene**

EC Number: **202-705-0**

CAS Number: **98-83-9**

The proposal was submitted by **Germany** and received by RAC on **18 March 2022.**

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

PROCESS FOR ADOPTION OF THE OPINION

Germany has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **25 April 2022**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **24 June 2022**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Michal Martínek**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **16 March 2023** by **consensus**.

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	601-027-00-6	2-phenylpropene; α-methylstyrene	202-705-0	98-83-9	Flam. Liq. 3 Eye Irrit. 2 STOT SE 3 Aquatic Chronic 2	H226 H319 H335 H411	GHS02 GHS09 GHS07 Wng	H226 H319 H335 H411		STOT SE 3; H335: C ≥ 25 %	
Dossier submitters proposal	601-027-00-6	2-phenylpropene; α-methylstyrene	202-705-0	98-83-9	**Add** Carc. 2 Skin Sens. 1B	**Add** H351 H317	**Add** GHS08	**Add** H351 H317			**Add** D
RAC opinion	601-027-00-6	2-phenylpropene; α-methylstyrene	202-705-0	98-83-9	**Add** Carc. 2 Skin Sens. 1B	**Add** H351 H317	**Add** GHS08	**Add** H351 H317			**Add** D
Resulting Annex VI entry if agreed by COM	601-027-00-6	2-phenylpropene; α-methylstyrene	202-705-0	98-83-9	Flam. Liq. 3 Carc. 2 STOT SE 3 Eye Irrit. 2 Skin Sens. 1B Aquatic Chronic 2	H226 H351 H335 H319 H317 H411	GHS02 GHS07GHS08 GHS09 Wng	H226 H351 H335 H319 H317 H411		STOT SE 3; H335: C ≥ 25 %	D

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

2-Phenylpropene (α-methylstyrene) is a volatile liquid (boiling point 165 °C, vapour pressure 253 Pa at 20°C). The substance is used as a co-monomer in a range of polymerisation processes, e.g. in the production of resins, paints, adhesives and plasticizers.

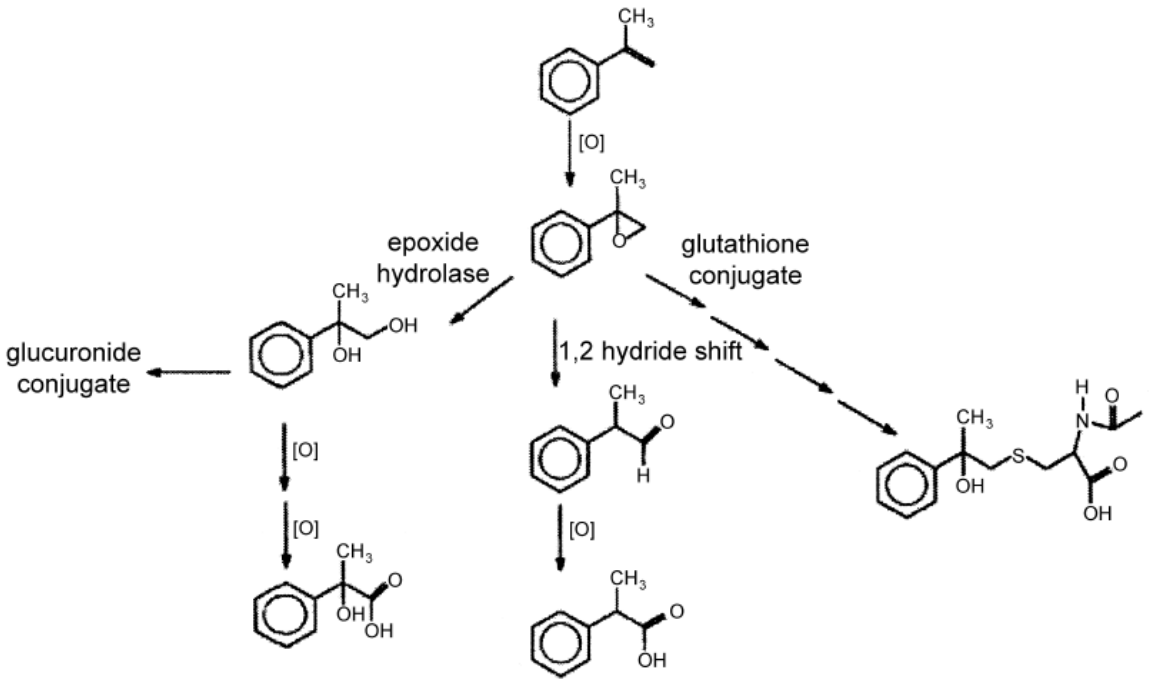
Note D

The dossier submitter (DS) proposed to add Note D because polymerisation inhibitor 4-*tert*-butylpyrocatechol is used (at 10-20 ppm) in the commercial formulations. Note D reads: *Certain substances which are susceptible to spontaneous polymerisation or decomposition are generally placed on the market in a stabilised form. It is in this form that they are listed in Part 3. However, such substances are sometimes placed on the market in a non-stabilised form. In this case, the supplier must state on the label the name of the substance followed by the words 'non-stabilised'.*

RAC agrees with the DS's proposal to **add Note D**.

Metabolism of α-methylstyrene

In an ADME study with α-methylstyrene in male F344 rats by NTP (2007), around 90 % of the radiolabel recovered after inhalation exposure was excreted in urine. The most abundant urinary metabolites were a glucuronide conjugate of 2-phenyl-1,2-propanediol, atrolactic acid (2-hydroxy-2-phenylpropanoic acid) and a mercapturate. A diagram of the proposed metabolic pathway is shown below (from NTP, 2007).



The first step in the proposed pathway is oxidation to 2-methyl-2-phenyloxirane (also known as α-methylstyrene oxide). This side-chain epoxide was not found in the urine or blood of rats administered α-methylstyrene. However, the structure of the identified metabolites and the fact that an analogous metabolite (styrene-7,8-oxide) has been detected in many studies with styrene provide sufficient confidence that α-methylstyrene is metabolised via the side-chain epoxide. In addition, a decrease in hepatic glutathione was observed in a short-term mouse inhalation study

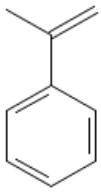
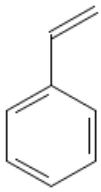
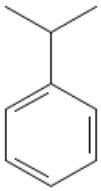
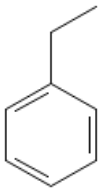
with α-methylstyrene (Morgan *et al.*, 1999; for details see 'supplemental information' in the carcinogenicity section), which is consistent with formation of a reactive intermediate in the liver.

Metabolites originating from hydrolysis and glutathione conjugation of α-methylstyrene oxide comprised about 85 % and 10 % respectively of the dose recovered from rat urine.

In an *in vitro* study in human liver slices from a single donor (De Costa *et al.*, 2001), 25 % of the radioactivity was present as 2-phenyl-1,2-propanediol, ≈1 % as atrolactic acid and ≈1 % as 2-phenylpropionic acid. The remainder of the metabolites accounted for less than 0.3 % of the radioactivity.

Structurally related substances

The DS used information from three structurally related substances, namely styrene, cumene and ethylbenzene, as supplementary information in the assessment of mutagenicity and carcinogenicity of α-methylstyrene. The structures of these substances are shown below.

α-methylstyrene	**styrene**	**cumene**	**ethylbenzene**
			

A brief overview of metabolism, genotoxicity, carcinogenicity and target organ toxicity of styrene, cumene and ethylbenzene can be found in the mutagenicity section under 'supplemental information'. The conclusions of the comparison can be summarised as follows.

- The main metabolic pathways of styrene are analogous to those of α-methylstyrene (side-chain epoxidation followed by hydrolysis or glutathione conjugation). Unlike α-methylstyrene, styrene is mutagenic *in vitro*, induces an increase in lung tumours in mice and causes severe hepatic necrosis in this species.
- Cumene is partly metabolised via α-methylstyrene in rats and mice and there are toxicological similarities between the two substances such as α2μ-globulin response in male rats and an increase in liver tumours in female mice. However, cumene additionally caused nasal tumours in rats and lung tumours in mice.
- The metabolism of ethylbenzene is not assumed to involve side-chain epoxidation. Despite some toxicological similarities (such as liver tumours in female mice) the dissimilar metabolism prevents the use of data on ethylbenzene in the assessment of α-methylstyrene.

In conclusion, data on styrene and cumene can be used to a limited extent in the assessment of α-methylstyrene, with due awareness of the significant toxicological differences. Use of data on ethylbenzene is not considered justified because of the dissimilarity of metabolic pathways.

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

The DS proposed to classify the substance as Skin Sens. 1B based on a positive LLNA giving an EC3 of 46 %.

Comments received during consultation

Two member state competent authorities (MSCAs) supported the DS's proposal.

Assessment and comparison with the classification criteria

In a GLP-compliant local lymph node assay (2016) according to OECD TG 429, female CBA/Ca mice (5/dose) were treated with α-methylstyrene in acetone/olive oil (4:1) at concentrations of 25 %, 50 % and 100 %. Hexyl cinnamic aldehyde (25 %) was used as a concurrent positive control. Stimulation indices at the individual concentrations are shown below.

Results of the LLNA (2016)	
Concentration	**Stimulation index**
0 %	1.00
25 %	2.35
50 %	3.13
100 %	4.50
Positive control (HCA 25 %)	6.08

The test is considered valid and the result is positive with an EC3 of 46 %. No other properly documented animal or human information on skin sensitisation can be found in the dossier.

With a positive reliable LLNA, the substance meets the criteria for classification. An EC3 of > 2 % corresponds to subcategory 1B. Thus, RAC agrees with the DS's proposal of **Skin Sens. 1B**.

RAC evaluation of specific target organ toxicity – repeated exposure (STOT RE)

Summary of the Dossier Submitter's proposal

Repeated dose toxicity of α-methylstyrene has been investigated in 3-month and 2-year inhalation studies in rats and mice and in an oral study in rats according to OECD TG 422. Several non-guideline studies are also available.

The kidney has been identified as the main target organ in rats. The renal effects were not considered sufficient for classification by the DS mainly because the changes below the guidance value (GV) were of low severity and some of them are likely to be related to α2μ-globulin nephropathy (male rat-specific phenomenon). Nasal lesions were the most sensitive effect upon

inhalation exposure in mice but the effects below the GV were also of a rather low severity. The DS further discussed findings in the liver and the thymus.

The DS concluded that criteria for a STOT RE classification are not met.

Comments received during consultation

Comments were received from 1 MSCA, who pointed out the high incidence (in nearly all animals) of nasal lesions (atrophy, metaplasia) in the mouse study. They were of the view that, despite their low severity, the findings should be considered as significant toxic effects warranting classification as STOT RE 2. The DS acknowledged that the nasal lesions at 75 and 150 ppm might represent a borderline case. Still, they retained their proposal of no classification due to the low severity and the existing classification as STOT SE 3; H335, which at least partly covers the findings in the respiratory tract.

Assessment and comparison with the classification criteria

Effects in the key studies are summarized in Table 27 of the CLH report. The RAC assessment focuses on effects below the (extrapolated) GVs.

3-to 12-day inhalation studies in rats and mice (Morgan et al., 1999)

These short-term studies in F344 rats and B6C3F1 mice reported hyaline droplet accumulation in the kidneys of male rats, increased liver weight in both species without a histopathological correlate and decreased spleen weight in mice. No histopathological changes were observed in the nasal cavity of mice. Further details can be found under 'supplemental information' in the carcinogenicity section. However, the shorter-term studies are considered less informative in relation to STOT RE classification than the available 3-month studies via the same route (studies of longer duration are generally given more weight in the classification, according to the Guidance on the application of the CLP criteria, version 5.0 (2017), section 3.9.2.3.2).

3-month inhalation studies in rats and mice (NTP, 2007)

F344 rats (10/sex/group) and B6C3F1 mice (10/sex/group) were exposed to α-methylstyrene via inhalation (whole body) for 14 weeks (6 hours/day, 5 days/week) at 0, 75, 150, 300, 600 and 1 000 ppm.

The effects at 150 and 75 ppm are below the GV for classification in category 2 (the 90-day inhalation study GV is 250 ppm).

The following effects were observed at 150 ppm in the 3-month rat study:

- Increased liver weight in males (relative by 10%)
- Increased α2μ-globulin and increased kidney cell proliferation (measured by a labelling technique) in males

RAC agrees with the DS that these findings are not sufficiently severe to warrant classification.

As to the mouse study, the histopathological changes in the nasal tissues are presented in the table below.

Nasal lesions in the 3-month mouse inhalation study (NTP, 2007)						
Concentration (ppm)	**0**	**75**	**150**	**300**	**600**	**1 000**
Males						
No. of males per group	10	10	10	10	10	10
Bowman's glands atrophy: incidence (mean severity score)	0	7** (1.0)	10** (1.3)	10** (1.9)	10** (2.0)	10** (2.0)
Bowman's glands hyperplasia (mean severity score)	0	9** (1.1)	10** (1.6)	10** (2.3)	10** (2.9)	10** (2.7)
Olfactory epithelium atrophy (mean severity score)	0	10** (1.1)	10** (1.4)	10** (2.0)	10** (2.0)	10** (2.1)
Olfactory epithelium metaplasia (mean severity score)	0	5* (1.2)	10** (1.4)	10** (2.0)	10** (2.0)	10** (2.0)
Respiratory epithelium hyaline degradation (mean severity score)	0	1 (1.0)	2 (1.0)	1 (1.0)	2 (1.0)	0
Females						
No. of females per group	10	10	10	10	10	10[a]
Bowman's glands atrophy (mean severity score)	0	8** (1.0)	9** (1.3)	10** (2.0)	10** (2.0)	8** (2.5)
Bowman's glands hyperplasia (mean severity score)	0	5* (1.0)	10** (1.7)	10** (2.3)	10** (2.6)	8** (2.6)
Olfactory epithelium atrophy (mean severity score)	0	10** (1.0)	10** (1.6)	10** (2.0)	10** (2.0)	8** (2.0)
Olfactory epithelium metaplasia (mean severity score)	0	4* (1.0)	9** (1.7)	10** (2.0)	10** (2.0)	8** (2.0)
Olfactory epithelium necrosis (mean severity score)	0	0	0	0	0	2 (3.0)
Respiratory epithelium hyaline degradation (mean severity score)	0	2 (2.0)	6** (1.3)	9** (1.6)	8** (1.4)	4* (1.0)

Statistically significant difference from control: *, $p \leq 0.05$; **, $p \leq 0.01$

Severity scores: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked

[a] Two top concentration females died before exposure on day 3; they had necrosis of the olfactory epithelium

The substance affected mainly the olfactory epithelium and virtually all animals were affected already at the lowest concentration tested (75 ppm). Atrophy and metaplasia of olfactory epithelium are generally significant toxic effects that may warrant classification. However, in this case the severity at relevant concentrations (150 ppm and below) was relatively low (minimal to mild; cf. the criteria of "significant organ damage" or "marked organ dysfunction" according to CLP, Annex I, 3.9.2.7.3) and no substantial increase in severity was observed at higher levels. A STOT RE classification for respiratory tract effects is therefore not considered justified.

Reproductive toxicity screening in rats according to OECD TG 422 via oral route (1997)

CD(SD) rats (10/sex/group) were administered α-methylstyrene in olive oil via gavage at 0, 40, 200 and 1 000 mg/kg bw/d. Males were dosed for 43 days, females for up to 53 days (14 days prior to mating and then throughout mating and gestation until termination on lactation day 4). The following effects were observed at the mid-dose of 200 mg/kg bw/d, which is around the extrapolated GV for classification in Category 2:

- Liver: increased liver weight in females, increased ALT in males, acidophilic change in hepatocytes (both sexes)
- Kidney: increased hyaline droplets and basophilic change of the renal tubular epithelium in males, vacuolation of the renal tubular epithelium in females (vacuoles identified as lipid droplets)
- Thymus: thymus atrophy in females

Further details (incidence, severity, magnitude of increases) are not provided in the CLH report.

The 'acidophilic change' in the liver was described as micro-granular acidophilic cells around the centre of the lobules (females) or diffusely spread (males), in males associated with loss of fatty droplets. Based on the available description, the liver findings are not considered sufficient for a STOT RE classification.

Hyaline droplets in male rats are probably related to accumulation of α2μ-globulin, an effect specific to male rats. Basophilic change of the renal tubular epithelium may indicate renal damage, possibly related to an additional mode of action besides α2μ-globulin nephropathy. Still, the kidney effects at 200 mg/kg bw/d are not considered sufficient for a STOT RE classification.

The severity of thymus atrophy in females at 200 mg/kg bw/d is not described in the CLH report. In general, thymus atrophy may represent a specific toxic effect or a non-specific response to stress. As there was no effect on thymus weight or histopathology in males in this study up to 1000 mg/kg bw/d, and no effect on the thymus was reported to occur in the inhalation studies by NTP (2007), it is uncertain whether the substance specifically affects this organ.

Conclusion

Kidney is the main target organ of α-methylstyrene in rats. However, the effects below the GVs are not sufficient for classification.

Nasal lesions represent the most sensitive effect in the mouse inhalation studies. Although atrophy of olfactory epithelium is generally a significant toxic effect, the severity of the finding at relevant concentrations was low in this case and no substantial increase in severity was observed at higher levels. Therefore, a STOT RE classification for respiratory tract effects is not considered justified.

In conclusion, RAC agrees with the DS's proposal of **no classification for STOT RE**.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

A standard *in vitro* battery consisting of several Ames tests, chromosomal aberration assays and an HPRT assay was negative. *In vitro* sister chromatid exchange (SCE) assays gave positive results. The presumed metabolic intermediate α-methylstyrene oxide was positive in an Ames test.

Two *in vivo* studies are available. A single dose oral bone marrow micronucleus (MN) assay in male mice (Rim *et al.*, 2012) was negative, whereas a peripheral blood MN assay integrated into the 3-month mouse inhalation study by NTP (2007) was positive in females.

The DS mentioned a number of factors to be considered in the classification, such as:

- The negative *in vitro* mutagenicity assays.
- The problematic interpretation of SCE results in relation to mutagenicity classification.
- The markedly differing protocols of the two *in vivo* MN assays. As a result, the negative oral MN test does not overrule the positive result of the inhalation study.
- The high general toxicity (including mortality) in the group of female mice showing increased MN frequencies.
- The unknown *in vivo* relevance of the presumed genotoxic metabolite.

According to the DS, the positive result in female mice in the inhalation study might warrant classification in Category 2. Still, in view of the significant uncertainties they preferred no classification due to inconclusive data.

Comments received during consultation

Comments were received from 2 MSCAs and 1 industry organisation.

The commenting MSCAs generally supported the DS's analysis and proposal for no classification, however, they also considered there is data available that might support Cat 2 classification, considering e.g. positive result found in female mice (but only in one study and one sex, the absence of a positive control and the high number of NCE also in the negative control). The other MN assay cannot be used to dismiss the positive results since the protocols are not similar with respect of duration of exposure, route of exposure and sex used i.e. male mice, that seem less sensitive than females.

Furthermore, in vitro SCE assays in the presence of S9, even of various qualities, are consistently positive and one of them with a clear dose-response, although in vitro mutagenicity assays negative. Also the toxicokinetics considerations point to the formation of an epoxide, with epoxides being highly reactive substances.

Particularly, one of the MSCAs requested QSAR analysis of the parent and metabolites, considering that metabolites are very similar to styrene glycol and styrene glycol glucuronide, which could in their view together with existing data possibly support a Cat. 2 classification. The DS confirmed that formation of α-methylstyrene oxide as the first metabolic step is predicted to occur in humans according to Meteor (Lhasa Limited). Still, the DS reiterated that relevance of this Ames-positive metabolite for *in vivo* mutagenicity of α-methylstyrene remains obscure.

The industry commenter supported no classification for mutagenicity and informed about their intention to conduct a new *in vitro* MN test in human whole blood. According to industry, this study is needed because the existing *in vitro* chromosomal aberration tests do not comply with the current OECD guidelines. Industry further proposed that, subject to a negative outcome of the *in vitro* MN test, a new *in vivo* study be performed to clarify the positive finding in the *in vivo* MN test by NTP (2007).

Industry further commented on some specific points of the DS's assessment. Regarding the *in vivo* MN test by NTP, they questioned biological plausibility of the observed increase in micronucleated normochromatic erythrocytes (MN-NCE) without a concomitant increase in micronucleated polychromatic erythrocytes (MN-PCE). Industry also challenged the use of data from styrene, cumene and ethylbenzene in the assessment of α-methylstyrene, pointing out the toxicological dissimilarities (e.g. different spectrum of neoplastic responses, positive Ames tests

with styrene). They claimed that although α-methylstyrene oxide is likely to be formed *in vivo*, the negative Ames tests with α-methylstyrene together with the fact that the epoxide was not detected in the ADME study indicate that it is rapidly converted to non-reactive downstream metabolites or conjugates.

During the third-party consultation, Industry informed about their intention to conduct a new *in vitro* micronucleus test in human whole blood. The experimental part of this study started in November 2022 and the audited draft report was received by ECHA in January 2023. The study is summarised below.

In vitro micronucleus assay in human lymphocytes (Gilby, 2023)

The study was conducted according to OECD TG 487 (2016) and under GLP. Alpha-methylstyrene (purity 99.83%) was tested in human lymphocyte cultures prepared from the pooled blood of two adult donors.

Details of the method:

- Vehicle: DMSO
- Metabolic activation: S9 from livers of β-naphthoflavone/phenobarbital-induced male Sprague Dawley rats
- Mitogen stimulation: phytohaemagglutiinin, 48 hours
- Short treatment ±S9: "3+17", i.e. a 3-hour treatment followed by removal of test article and addition of cytochalasin B (cytoB); sampling at 20 hours after the beginning of treatment
- Extended treatment –S9: "20+0", i.e. exposure to the test chemical and cytoB for 20 hours, followed by sampling
- Replicates: duplicate cultures for each treatment level and positive control, quadruplicate cultures for vehicle controls
- Positive controls: Mitomycin C as a clastogenic control −S9, Colchicine as an aneugenic control −S9, Cyclophosphamide as a clastogenic control +S9
- Basis for top concentration selection: cytotoxicity
- Cytotoxicity parameter: cytokinesis-block proliferation index (CBPI)
- Number of analysed concentrations: 3 concentrations per treatment schedule selected for micronucleus analysis. The highest concentrations selected for analysis were those at which 50-60% cytotoxicity was achieved.
- Number of cells analysed for micronuclei: 2000 binucleated cells per concentration and 4000 cells for the vehicle control
- Method of analysis: fluorescence microscopy

All acceptability criteria (as specified in paragraph 56 of the OECD TG) were met. The results are summarised in the following table.

***In vitro* micronucleus test in human lymphocytes (Gilby, 2023)**					
Treatment	**Concentration (µg/ml)**	**Cytotoxicity (%, based on CBPI)**	**Mean MN cell frequency (%)**	**Historical control range (95th percentile)**	**Statistical significance**
3+17 –S9	Vehicle	–	0.38	0.20–1.00	–
	37.2	3	0.60		NS
	102	17	0.35		NS
	126	51	0.10		NS
	MMC, 0.3	33	2.00	1.50–6.08	p≤0.001
	COL, 0.07	21	1.95	1.48–3.70	p ≤ 0.001

3+17 +S9	Vehicle	–	0.65	0.20–1.10	–
	60	2	0.75		NS
	135	29	0.50		NS
	150	52	0.65		NS
	CPA, 10.0	54	1.90	1.21–2.59	$p \leq 0.01$
20+0 –S9	Vehicle	–	0.48	0.20–1.00	–
	18.6	0	0.40		NS
	82.7	25	0.50		NS
	113	52	0.45		NS
	MMC, 0.1	20	2.70	1.35–3.65	$p \leq 0.001$
	COL, 0.02	12	1.70	1.20–2.36	$p \leq 0.001$

MMC = Mitomycin C; COL = Colchicine; CPA = Cyclophosphamide; NS = no significant increase

It is concluded that alpha-methylstyrene did not induce micronuclei in cultured human peripheral blood lymphocytes when tested up to the cytotoxicity limit prescribed by the respective OECD test guideline, in both the absence and presence of metabolic activation.

Assessment and comparison with the classification criteria

In vitro assays

The available *in vitro* assays with α-methylstyrene are summarised in the table below. Detailed descriptions of the *in vitro* mutagenicity assays were not available to RAC except the studies by NTP (2007) and the new micronucleus test (Gilby, 2023).

***In vitro* genotoxicity assays with α-methylstyrene**			
Study type; year / reference	**Method**	**Result**	**Remarks**
Ames test 1997	Plate incorporation method Rat liver S9 Top concentration 400 µg/plate Solvent DMSO	Negative ±S9 Cytotoxicity from 200 µg/plate	
Ames test NTP, 2007	Pre-incubation method Rat and hamster liver S9 Top concentration 100 to 3 333 µg/plate	Negative ±S9 Cytotoxicity from 333–3 333 µg/plate (depending on strain and metabolic activation)	TA102 or *E.coli* WP2 not tested
Ames test 1991	Rat liver S9 Top concentration 1 000 µg/plate Solvent DMSO	Negative ±S9 Cytotoxicity from 100 µg/plate	TA102 or *E.coli* WP2 not tested
Ames test 1989	Rat liver S9 Top concentration 1 000 µg/plate Solvent acetone	Negative ±S9 Cytotoxicity from 100 µg/plate	TA102 or *E.coli* WP2 not tested
Chromosomal aberrations	Chinese hamster lung cells Rat liver S9	Negative ±S9	

***In vitro* genotoxicity assays with α-methylstyrene**			
Study type; year / reference	**Method**	**Result**	**Remarks**
1997	Top concentration 170 to 230 µg/mL Solvent DMSO	Cytotoxicity from 170 µg/mL	
Chromosomal aberrations NTP, 2007	Chinese hamster ovary cells Rat liver S9 Top concentration 200 µg/mL Solvent DMSO	Negative ±S9 Cytoxicity at 250 µg/mL	Relatively short exposure (+S9 2 h) and sampling time (10-12 h)
Chromosomal aberrations 1991	Chinese hamster ovary cells Rat liver S9 Top concentration 0.15 µL/mL Solvent DMSO	Negative ±S9 Cytotoxicity from 0.1 µL/mL	
Micronucleus Gilby, 2023	Human lymphocytes Rat liver S9 Highest analysed concentration 113 to 150 µg/mL Solvent DMSO	Negative ±S9 Cytotoxicity: CBPI reduction by > 50 % at the highest analysed concentration in each experiment	
HPRT test 1991	Chinese hamster ovary cells Rat liver S9 Top concentration 0.15 µL/mL Solvent DMSO	Negative ±S9 Excessive cytotoxicity above 0.1 µL/mL	
Sister chromatid exchange NTP, 2007	Chinese hamster ovary cells Rat liver S9 Top concentration 50 to 150 µg/mL Solvent DMSO	Positive +S9 with a dose response Cytotoxicity at 167 µg/mL	
Sister chromatid exchange Norppa and Vainio, 1983	Human lymphocytes (whole blood) No external metabolic activation Solvent acetone	Weakly positive	12 substances tested Results in graphical form, confusing presentation of data The authors assumed that styrene and related compounds were converted to a reactive metabolite by erythrocytes present in the *in vitro* system
Sister chromatid exchange Norppa and Tursi, 1984	Human lymphocytes (whole blood) No external metabolic activation Top concentration 2 mM (236 µg/mL)	Positive	

The DS further presented an Ames test with α-methylstyrene oxide by Rosman *et al.* (1986). The authors investigated mutagenicity of styrene oxide, α-methylstyrene oxide and related compounds in *Salmonella* strains TA1535 and TA100 using the pre-incubation method. A strong and reproducible dose-related increase in revertants was observed for α-methylstyrene oxide in TA100 at non-cytotoxic concentrations, whereas only a marginal activity was seen in TA1535. Styrene oxide tested positive in TA100.

Ames tests with α-methylstyrene were negative. The lack of investigations in TA102 or *E.coli* WP2 in some of the studies is not considered critical given that the metabolite α-methylstyrene oxide and the structurally related substance styrene tested positive in TA100 and/or TA1535.

In vitro chromosomal aberration assays were negative but some deviations from the current version of OECD TG 473 are noted, such as short harvest time and lower number of scored cells. Such deviations generally decrease assay sensitivity.

The recently submitted *in vitro* micronucleus test in human lymphocytes, conducted according to OECD TG 487 (2016) without deviations, is negative.

A gene mutation test in mammalian cells (HPRT test) was negative. A detailed description of the study is not available to RAC.

A positive result was obtained with metabolic activation in the SCE assay by NTP (2007). However, SCE tests are generally given lower weight than mutagenicity tests in the classification.

To sum up, α-methylstyrene was negative in a battery of standard *in vitro* mutagenicity tests. The substance tested positive for sister chromatid exchange.

In vivo assays

The two available *in vivo* tests with α-methylstyrene are summarised in the following table.

***In vivo* genotoxicity assays with α-methylstyrene**			
Study type; year / reference	**Method**	**Result**	**Remarks**
Micronucleus, mouse, peripheral blood, integrated into a 3-month inhalation study NTP, 2007	B6C3F1 mice 10/sex/group Inhalation (whole body), 14 weeks (6 hours/day, 5 days/week) Concentrations: 0, 75, 150, 300, 600, 1 000 ppm 1000 NCEs and 1000 PCEs per animal No concurrent positive control	NCEs: males negative, females positive at 1 000 ppm PCEs: negative in both sexes	Concurrent negative controls above HCD range No bone marrow toxicity General toxicity at 1 000 ppm included mortality (2 females before exposure on day 3), clinical signs of toxicity (moderate to severe sedation in males, ataxia in both sexes) and target organ effects (liver, nose)
Micronucleus, mouse, bone marrow, oral (gavage) Rim *et al.*, 2012	ICR mice 6 males/group Doses: 0, 500, 1 000, 2 000 mg/kg bw Vehicle olive oil Single exposure Sampling at 24 h 2000 PCEs per animal	Negative in males (females not tested)	No bone marrow toxicity No clinical signs of toxicity

NCE = normochromatic erythrocyte; PCE = polychromatic erythrocyte

The results of the MN test by NTP, integrated into a 3-month study, are shown in detail in the table and the graph below. HCD and individual data have been obtained from the NTP online database (NTP, 2022a).

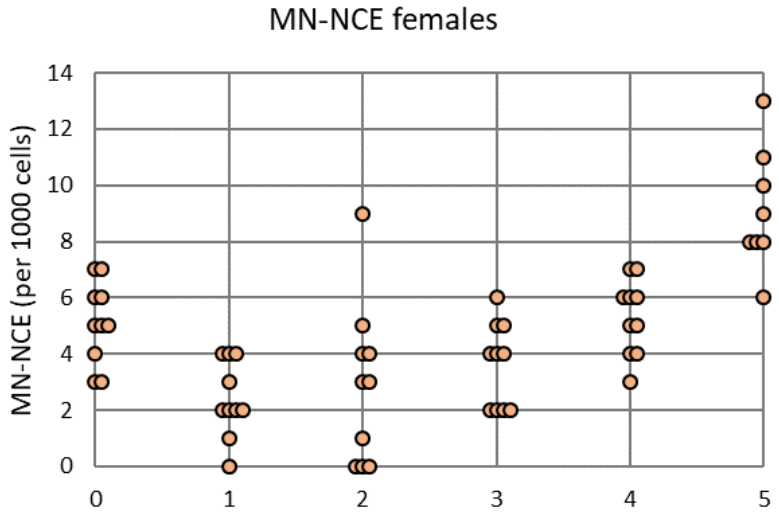
Mouse micronucleus test in the 3-month inhalation study (NTP 2007)				
Concentration (ppm)	**Micronucleated cells/1000 cells**			
	Males		**Females**	
	PCEs	**NCEs**	**PCEs**	**NCEs**
0	3.9	5.3	4.1	5.1
75		5.8		2.4
150		5.8		2.9
300		5.0		3.6
600		4.6		5.3
1000	5.0	6.3	4.8	9.1*
HCD inhalation[a]		mean 1.6 SD 0.9 max. 3.4		mean 1.4 SD 1.0 max. 4.0
HCD all routes[b]			mean 2.4 SD 1.1 max. 4.5	mean 1.3 SD 0.9 max. 4.2

NCE = normochromatic erythrocyte; PCE = polychromatic erythrocyte; SD = standard deviation

* Statistically significant difference on pairwise comparison, $p \leq 0.025$

[a] 17 three-month NTP inhalation studies in B6C3F1 mice (peripheral blood, slide scoring) conducted between 1996 and 2005 (current study 2000); the maximum values represent group mean (not individual data)

[b] MN-NCE: 69 three-month studies in female B6C3F1 mice conducted between 1996 and 2005; MN-PCE: 14 three-month studies in female B6C3F1 mice conducted between 1996 and 2005



Numbering of groups: 0 = control, 1 = 75 ppm, 2 = 150 ppm, 3 = 300 ppm, 4 = 600 ppm, 5 = 1000 ppm

The study did not strictly follow OECD TG 474. One of the deviations is that the test design does not include positive control. Nevertheless, the protocol has been shown to be able to generate positive results (e.g. the in the 3-month gavage study with butanal oxime conducted in 1996; NTP, 2004), so the lack of positive control is not considered critical in this case. Another deviation is that the concurrent control values are outside (above) the historical control distribution. This

introduces some uncertainty. Lastly, the top concentration induced high general toxicity in both sexes: 2 out of 10 females died before exposure on day 3, moderate to severe sedation was observed in males.

It is noted that there was no significant increase in micronucleated polychromatic (immature) erythrocytes (MN-PCE) in top concentration females ($p = 0.26$).

Overall, the NTP MN test is considered positive in females in the presence of excessive toxicity. The positive result is associated with uncertainties such as abnormally high concurrent control value and lack of a significant increase in micronucleated immature erythrocytes.

The negative oral single-dose bone marrow micronucleus test in male ICR mice by Rim *et al.* (2012) appears well-conducted. Still, the design shows some deviations from the OECD TG. Generally, repeated administration is preferable to single treatment and when a single treatment is used, bone marrow samples should be taken at least twice (e.g. at 24 and 48h) in order not to miss the peak MN frequency. Further, testing of females should have been considered given the higher sensitivity of this sex in earlier studies (NTP, 2007; Morgan *et al.*, 1999).

Thus, there is an *in vivo* micronucleus test positive in one sex (females) above the maximum tolerated concentration. The other test is negative but the more susceptible sex (females) was not tested and the study has deficiencies potentially reducing its sensitivity.

Genotoxicity of structurally related substances

Styrene:

- *In vitro:* The majority of Ames tests were negative but some positive results have been reported in the presence of metabolic activation. *In vitro* aberration tests (having limitations) were positive, an HPRT assay was negative.
- *In vivo:* Reliable chromosomal aberration and micronucleus assays via physiological routes (oral, inhalation) were negative, via i.p. route negative (2 studies) or positive (1 study, positive only at a dose causing high mortality). A standard comet assay via inhalation in lymphocytes was negative, a positive result was obtained in the same study when Fpg was employed (Fpg modification of comet assay enhances detection of certain types of DNA damage such as base oxidation).
- Human data: Various genotoxicity endpoints have been investigated in workers exposed to styrene, some positive associations have been reported.
- RAC opinion: Mutagenicity of styrene has not been evaluated by RAC.

Cumene:

- *In vitro:* The substance was not mutagenic in bacteria. An *in vitro* chromosomal aberration assay was negative but had deficiencies. Inconclusive results were obtained for gene mutation in mammalian cells due to study limitations.
- *In vivo:* MN tests via physiological routes (oral, inhalation) were negative, via the i.p. route positive. A comet assay in rats and mice via oral route investigating the liver, lungs, kidney and blood leukocytes was negative in all tissues except male rat liver and female mouse lung.
- RAC opinion (2020): RAC concluded that although a weak genotoxic potential of cumene cannot be excluded, the criteria for classification in Category 2 are not fulfilled.

Conclusion

In the absence of evidence of germ cell mutagenicity in humans, any positive *in vivo* germ cell mutagenicity test(s) in animals or evidence that the substance or its metabolite interact with the genetic material of germ cells, the criteria for classification in Category 1 are not met.

Classification in Category 2 is based on positive evidence obtained from experiments in mammals and/or in some cases from *in vitro* experiments, obtained from (a) *in vivo* somatic cell mutagenicity tests in mammals, or (b) other *in vivo* somatic cell genotoxicity tests which are supported by positive results from *in vitro* mutagenicity assays. Where a single well-conducted test is used for classification, it shall provide clear and unambiguously positive results (CLP, Annex I, 3.5.2.3.9).

In this case the *in vitro* mutagenicity database is negative. *In vitro* sister chromatid exchange assays are positive but these are given lower weight than mutagenicity assays and are not mentioned in the classification criteria nor in the examples in CLP, Annex I, 3.5.2.3. The *in vivo* database consists of two micronucleus tests. One is negative but has deficiencies potentially decreasing its sensitivity. The other one is positive in females (only in normochromatic erythrocytes) and negative in males. The positive result in females is associated with uncertainties such as excessive general toxicity and absence of a concomitant increase in micronucleated polychromatic erythrocytes.

The available evidence indicates that α-methylstyrene is metabolised via an Ames-positive epoxide. Nevertheless, the classification criteria are mainly based on evidence for the parent substance. The *in vivo* levels of the reactive intermediate might be low depending on the rate of formation and detoxification.

Given the negative *in vitro* mutagenicity database and the uncertainties related to the single positive *in vivo* result, RAC agrees with the DS that the available evidence **does not meet the criteria and recommends no classification for germ cell mutagenicity**.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

Two carcinogenicity studies via inhalation are available, one in rats and one in mice (NTP, 2007). The DS proposed classification in Category 2 based on kidney tumours in male rats and liver tumours in female and male mice. According to the DS, the evidence is not sufficient for a 1B classification because the kidney tumours in male rats appear to be partly related to α2μ-globulin accumulation (a species-specific phenomenon), B6C3F1 mice have a high background incidence of liver tumours, there is no strong evidence of genotoxicity, and the pattern of liver effects (increased weight, centrilobular hypertrophy, eosinophilic foci, no cytotoxicity) suggests CAR activation. The DS did not propose a specific concentration limit as the T25 values correspond to medium or low potency.

Comments received during consultation

Comments were received from 2 MSCAs and 1 industry organisation.

While industry supported the DS's proposal of Category 2, the commenting MSCAs preferred Category 1B, putting forward the following arguments:

- Clear carcinogenic effects are present in both sexes, in two species and also multi-site responses
- There is no strong evidence for a rodent-specific mode of action (MoA) of the liver tumours observed in mice, therefore human relevance has to be assumed.
- The increase in liver tumours in male mice, although less pronounced than in females, is significant and has to be considered in the classification.

- Increases in mononuclear cell leukemia and testicular tumours / adenomas above HCD observed in male rats are treatment-related and relevant for classification.
- Neoplastic effects in the liver and kidney may be related to local formation of a reactive metabolite.
- Structurally related substances are carcinogenic: cumene has a harmonized classification as Carc. 1B, styrene has been classified by IARC in group 2A, which is considered equivalent to Carc. 1B.

In response to these comments the DS pointed out the following:

- The increase in interstitial cell adenomas was considered unrelated to treatment by the study authors because the incidences only slightly exceeded the historical control range, there was no clear concentration dependency, the concurrent control was below the historical control range and testicular adenomas are very common in aged F344 rats.
- Human relevance of mononuclear cell leukemia has been questioned due to spontaneous occurrence in aged F344 rats with variable and high incidence, species-specific characteristics, mechanistic considerations and reproducibility issues.
- As to the liver tumours in male mice, statistically significant effects were only seen when incidences of adenomas and carcinomas were combined, there was no clear concentration dependence and the incidences only slightly exceeded the historical control range.
- In contrast to cumene, lung tumours in mice and nasal tumours in rats have not been observed with α-methylstyrene.

Assessment and comparison with the classification criteria

2-year carcinogenicity studies in rats and mice (NTP, 2007): general information

F344 rats (50/sex/group) were exposed to α-methylstyrene vapours (whole body, 6 h/day, 5 days/week) at 0, 100, 300 and 1 000 ppm. The top concentration selection was based on the 3-month study where some but not excessive toxicity was observed at 1 000 ppm. Generation of concentrations above 1000 ppm was reported to result in production of unwanted aerosols (Morgan *et al.*, 1999). General toxicity in the 2-year rat study included a mild body weight reduction (< 10 %) and nasal lesions such as olfactory epithelium degeneration of minimal severity. Survival was not affected. The following tumour types showed a statistically significant increase compared to concurrent control in males: renal tubular adenoma and carcinoma (combined), mononuclear cell leukaemia, and testicular adenoma. No neoplastic effects were detected in females.

B6C3F1 mice (50/sex/group) were exposed to α-methylstyrene vapours at 0, 100, 300 and 600 ppm. The top concentration selection was based on the 3-month study where excessive toxicity including mortality occurred at 1000 ppm. General toxicity at the top concentration in the 2-year study included body weight reduction (by ca. 10-15 %) and nasal lesions (olfactory epithelium metaplasia and atrophy). Survival was not affected. Increased incidence of hepatocellular adenomas and carcinomas was observed in females, a marginal increase in carcinomas also occurred in males.

The NTP report contains historical control data comprising inhalation studies beginning within six years before the current studies (1995-2000, the 2-year studies with α-methylstyrene started in July/August 2001). To make the best use of the available information, RAC compiled historical control data from the NTP online database (NTP, 2022b), including also studies conducted after the current study. The usual rules for HCD have been followed (±5 years of the current study, the same strain, source of animals, laboratory, study duration, exposure route and diet). Further details can be found under 'supplemental information'.

Kidney tumours in male rats

Neoplastic and non-neoplastic kidney findings in male and female rats from the 2-year inhalation study (NTP, 2007) are presented in the table below. Initially, a single hematoxylin and eosin-stained section of each kidney was prepared. Because of the neoplastic and non-neoplastic renal findings and a possible mode of action (MoA) via α2μ-globulin accumulation, additional kidney step sections (three to four from each kidney) were prepared from the remaining formalin-fixed tissues at 1 mm intervals for each male. This led to identification of additional males with focal hyperplasia or adenoma.

2-year inhalation study in rats: kidney histopathology					
Concentration (ppm)	**0**	**100**	**300**	**1 000**	**HCD[a]**
Males					
No. of males examined	50	50	50	50	
Papilla, mineralization; incidence (mean severity)	12 (1.1)	16 (1.0)	10 (1.0)	33** (1.4)	
Nephropathy (mean severity)	41 (2.2)	46 (2.3)	46 (2.4)	45 (2.4)	
Renal tubule hyperplasia (single sections)	0	0	0	0	
Renal tubule adenoma (single sections)	0	0	1 (2 %)	0	Mean±SD 0.8 %±1.0 % Range 0-2 %
Renal tubule carcinoma (single sections)	0	0	1 (2 %)	2 (4 %)	Mean±SD 0.3 %±0.8 % Range 0-2 %
Renal tubule adenoma or carcinoma (single sections)	0	0	2 (4 %)	2 (4 %)	Mean±SD 1.2 %±1.3 % Range 0-4 %
Renal tubule hyperplasia (single and step sections)	1	0	1	4	
Renal tubule adenoma (single and step sections)	1	2	2	5	
Renal tubule carcinoma (single and step sections)	0	0	1	2	
Renal tubule adenoma or carcinoma (single and step sections)	1 (2 %)	2 (4 %)	3 (6 %)	7* (14 %)	
Renal tubule adenoma or carcinoma first incidence days	729	723	716	653	
Females					
No. of females examined	49	50	50	50	
Papilla, mineralization (mean severity)	1 (1.0)	6 (1.0)	8* (1.0)	7* (1.0)	
Pelvis, transitional epithelium, mineralization (mean severity)	31 (1.5)	26 (1.0)	31 (1.1)	16** (1.0)	
Nephropathy (mean severity)	34 (1.6)	27 (1.3)	35 (1.5)	31 (1.8)	

Statistically significant difference from control (Poly-3 test): *, $p \leq 0.05$; **, $p \leq 0.01$

Severity scores: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked

[a] 12 two-year studies via inhalation starting between 1997 and 2005 (the current study started in 2001); the same laboratory, source of animals and diet

There was a dose-dependent increase in renal tubule neoplasms in males, reaching statistical significance on pairwise comparison at 1 000 ppm for adenomas and carcinomas combined (single and step sections). Historical control data is only available for standard (single section) histopathological evaluation. Kidney was a target organ of α-methylstyrene toxicity also in studies of shorter duration (Morgan *et al.*, 1999; 3-month study by NTP, 2007; reproductive toxicity screening, 1997; for details see 'supplemental information'). The increase in renal tubular tumours is considered treatment-related.

The authors of the NTP report (2007) discussed the kidney findings in studies with α-methylstyrene in relation to the criteria established by IARC (1999) and US EPA (1991) for male rat kidney carcinogenicity through an α2μ-globulin -associated response. This mode of action is considered not relevant for humans (see the Guidance on the application of the CLP criteria, section 3.9.2.5.3, and a review by RIVM (RIVM, 2002).

α2μ-Globulin is a protein synthesized in the liver of male rats. It is filtered through the renal glomerulus, reabsorbed in the tubules and degraded by lysosomes of tubular cells. The sequence of events leading to tumours via α2μ-globulin -associated nephropathy can be summarised as follows: A chemical or its metabolite binds reversibly to α2μ-globulin. The protein-chemical complex is resistant to breakdown, leading to accumulation of the complex in lysosomes and cell death. Dead cells are sloughed into the lumen and contribute to the development of granular casts. Cell death and degeneration causes a compensatory cell proliferation in the cortex. Sustained cell proliferation leads to promotion of spontaneously initiated cells and formation of renal adenoma and carcinoma.

IARC (1999) developed the following set of criteria for concluding that an agent causes kidney tumours through an α2μ-globulin -associated response:

1. Lack of genotoxic activity (agent and/or metabolite) based on an overall evaluation of *in vitro* and *in vivo* data
2. Male rat specificity for nephropathy and renal tumorigenicity
3. Induction of the characteristic sequence of histopathological changes in shorter-term studies, of which protein droplet accumulation is obligatory
4. Identification of the protein accumulating in tubule cells as α2μ-globulin
5. Reversible binding of the chemical or metabolite to α2μ-globulin
6. Induction of sustained increased cell proliferation in the renal cortex
7. Similarities in dose-response relationship of the tumour outcome with the histopathological endpoints (protein droplets, α2μ-globulin accumulation, cell proliferation)

The characteristic sequence of histopathological changes referred to in point 3 includes excessive accumulation of hyaline droplets, single-cell necrosis of the tubule epithelium, regenerative cell tubule proliferation, development of intraluminal granular casts from sloughed cell debris (associated with tubule dilation and papillary mineralization), foci of tubule hyperplasia in the convoluted proximal tubules and renal tubule tumours (US EPA, 1991).

Information on α-methylstyrene related to the individual criteria can be summarised as follows:

1. Genotoxicity: The available data do not meet the criteria for mutagenicity classification. α-Methylstyrene is metabolized via a DNA-reactive intermediate (α-methylstyrene oxide) but its contribution to *in vivo* carcinogenicity is unknown since detoxification mechanisms such as enzymatic hydrolysis are likely to considerably reduce its levels in tissues.
2. Male rat-specific effect: An increase in renal neoplasms was only observed in male rats. However, evidence of renal toxicity was also seen in female rats, namely increased kidney weight and increased urinary markers of kidney toxicity in the 3-month study (for details see 'supplemental information'). In addition, an increase in nephropathy was observed in

female mice in the 2-year study (incidence 26/50 vs 16/50 in control, mean severity 1.6 vs 1.1).

3. Characteristic sequence of histopathological changes: The 3-month rat study reported a slight increase in hyaline droplet accumulation from 600 ppm. An increase in pelvic mineralization was observed in the 2-year study at 1 000 ppm. On the other hand, the 2-year rat study did not show a significant increase in nephropathy or hyperplasia in male animals.
4. Identification of the protein as α2μ-globulin: The amount of α2μ-globulin was determined in kidney homogenates of male rats in the 3-month study. A significant increase in α2μ-globulin per μg of soluble protein was observed from 150 ppm.
5. Binding of the substance or metabolites to α2μ-globulin: not investigated
6. Increased cell proliferation: Increased proliferation (starting from 150 ppm) was detected in male rats of the 3-month study using a labelling technique.
7. Similarity in dose-response response for neoplastic and non-neoplastic findings: A statistically significant increase in renal tumours (adenomas and carcinomas combined) was observed at 1 000 ppm. An increase in severity of hyaline droplets started around 600 ppm, papillary mineralization was increased at 1000 ppm. There was no increase in the severity or incidence of nephropathy up to the top concentration.

In line with the authors of the NTP report (2007) and the DS, RAC concludes that α-methylstyrene meets some, but not all, the criteria for male rat kidney carcinogenicity through an α2μ-globulin -associated response. The uncertainties are related to lack of investigations (binding of the substance and metabolites to α2μ-globulin, cell proliferation in female rats), inconsistency of some observations with the MoA (e.g. no increase in nephropathy in male rats) or indications of other possible MoAs (evidence of renal toxicity in female rats). Human relevance of the renal tumours can therefore not be completely excluded. Nevertheless, the concern is reduced by partial involvement of α2μ-globulin -related MoA and absence of renal neoplasms in females.

Mononuclear cell leukaemia in male rats

F344/N rats used in NTP studies had a high and gradually increasing spontaneous incidence of mononuclear cell leukaemia. Nevertheless, the statistically significant increase (Poly-3 p = 0.016) in top concentration males exceeds the relevant HCD. No significant increase was observed in females.

The latency may be slightly reduced at the top concentration compared to concurrent control. Spontaneous cases in control F344 rats generally begin to appear after 70 weeks of age (Caldwell, 1999). In this study the first case in top concentration males was observed in week 64 of age compared to week 77 in the control.

Mononuclear cell leukaemia in F344 rats has been reviewed by RIVM (Scheepmaker *et al.*, 2005). They noted that other rat strains and other species including humans have a much lower incidence, increased incidence in F344 rats had in some cases not been reproduced in a second study in F344 rats of a comparable design (e.g. acetaminophen, butyl benzyl phthalate) and had never been confirmed in another rat strain or in mice. Human relevance of an increase in mononuclear cell leukaemia observed only in F344 rats is generally considered low, although unresolved questions remain (Thomas *et al.*, 2007) and case-by-case evaluation is needed.

2-year inhalation study in rats: mononuclear cell leukaemia					
Concentration (ppm)	**0**	**100**	**300**	**1 000**	**HCD[a]**
Males					
No. of males examined	50	50	50	50	

Mononuclear cell leukaemia	26 (52 %)	32 (64 %)	29 (58 %)	38* (76 %)	Mean±SD 48 %±9 % Range 34-66 %
First incidence (days)	495	562	558	401	
Females					
No. of females examined	50	50	50	50	
Mononuclear cell leukaemia	18 (36 %)	21 (42 %)	21 (42 %)	22 (44 %)	Mean±SD 33 %±11 % Range 20-52 %

* Statistically significant difference from control (Poly-3 test), $p \leq 0.05$

[a] 12 two-year studies via inhalation starting between 1997 and 2005 (the current study started in 2001); the same laboratory, source of animals and diet

Testicular tumours in rats

F344 rats used in NTP studies had a high and variable incidence of interstitial cell adenomas. The incidences at 100 and 1 000 ppm in the current study were significantly different from concurrent control but there was no dose-response relationship (the incidence at 1 000 ppm is equal to that at 100 ppm). The historical control range was marginally exceeded. RAC agrees with the authors of the NTP report and with the DS that the increase in testicular adenomas may be incidental.

2-year inhalation study in rats: testicular tumours					
Concentration (ppm)	**0**	**100**	**300**	**1 000**	**HCD[a]**
No. of males examined	50	50	50	50	
Testes, adenoma	33 (66 %)	44* (88 %)	41 (82 %)	44* (88 %)	Mean±SD 74 %±8% Range 58-84 %

* Statistically significant difference from control (Poly-3 test), $p \leq 0.05$

[a] 12 two-year studies via inhalation starting between 1997 and 2005 (the current study started in 2001); the same laboratory, source of animals and diet

Liver tumours in male and female mice

B6C3F1 mice, particularly males, have a high spontaneous incidence of hepatocellular tumours. They are also particularly sensitive to induction of liver tumours by chemicals compared to some other strains of mice and to other species (see e.g. Scheepmaker *et al.*, 2005; Grisham, 1996).

Male mice in this study showed a significantly increased incidence of hepatocellular adenomas and carcinomas combined. It is noted that the top dose incidence remained close to the historical control mean.

Females showed statistically significant increases in hepatocellular adenomas, hepatocellular carcinomas, and adenomas and carcinomas combined. The incidence of carcinoma at the top concentration markedly exceeded the historical control range. There was a biologically plausible sequence of increased eosinophilic foci, adenoma and carcinoma. Part of the liver carcinomas metastasized to the lung (a common site of metastasis of liver tumours). No robust MoA information is available.

Short-term and subchronic inhalation studies with α-methylstyrene reported increased liver weight, hepatocellular hypertrophy and depletion of hepatic glutathione (see 'supplemental information'). Glutathione depletion indicates formation of a reactive metabolite in the liver. Whether this metabolite is responsible for the increase in tumours (e.g. via genotoxicity or cytotoxicity) is currently unknown. Hepatocellular hypertrophy may indicate a MoA via activation of nuclear receptors (e.g. CAR). However, no specific studies on activation of nuclear receptors

or other MoAs are available. Thus, the mode of action (or the combination of MoAs) leading to liver tumours in α-methylstyrene-exposed mice remains unknown.

As to the structurally related substances, cumene caused a weak increase in hepatocellular ademonas in female mice but not in male mice or in rats (NTP, 2009). No liver tumours were found in mice exposed to styrene (Cruzan *et al.*, 2001) but the top concentration had to be relatively low (160 ppm) due to mortality and severe toxicity (including liver necrosis) at higher exposure levels.

In summary, the increase in liver tumours in female B6C3F1 mice is clearly related to treatment and the increase in males may also have been caused by the substance. On the other hand, the concern is reduced by the high background incidence and the high susceptibility of this strain.

Liver tumours in mice					
Concentration (ppm)	**0**	**100**	**300**	**600**	**HCD[a]**
Males					
No. of animals examined	50	50	50	50	
Hepatocellular adenoma	24 (48 %)	27 (54 %)	27 (54 %)	25 (50 %)	Mean±SD 51 %±14 % Range 30-74 %
Hepatocellular carcinoma	10 (20 %)	12 (24 %)	11 (22 %)	17 (34 %)	Mean±SD 30 %±11 % Range 18-52 %
Hepatocellular adenoma or carcinoma	28 (56 %)	36* (72 %)	33 (66 %)	37* (74 %)	Mean±SD 67 %±13 % Range 50-88 %
Lung, metastatic hepatocellular carcinoma[b]	4 (8 %)	6 (12 %)	4 (8 %)	7 (14 %)	Mean±SD 11 %±6 % Range 0-20 %
Hepatocellular carcinoma first incidence (days)	549	537	565	429	
Females					
No. of animals examined	50	50	50	50	
Eosinophilic focus	2	5	7	12**	
Hepatocellular adenoma	10 (20 %)	20* (40 %)	21** (42 %)	23** (46 %)	Mean±SD 29 %±10 % Range 12-50 %
Hepatocellular carcinoma	3 (6 %)	9 (18 %)	6 (12 %)	18** (36 %)	Mean±SD 12 %±4 % Range 8-20 %
Hepatocellular adenoma or carcinoma	13 (26 %)	26** (52 %)	24* (48 %)	33** (66 %)	Mean±SD 38 %±11 % Range 22-56 %
Lung, metastatic hepatocellular carcinoma[b]	1 (2 %)	5 (10 %)	3 (6 %)	13 (26 %)	Mean±SD 6 %±3 % Range 0-10 %
Hepatocellular carcinoma first incidence (days)	634	537	416	612	

Statistically significant difference from control (Poly-3 test): *, $p \leq 0.05$; **, $p \leq 0.01$

[a] 13 two-year studies via inhalation starting between 1997 and 2006 (the current study started in 2001), the same laboratory, source of animals and diet

[b] Statistical analysis not conducted

Comparison with criteria

According to the CLP criteria, sufficient evidence of carcinogenicity in experimental animals, corresponding to Category 1B, is available when a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single species in a well-conducted study can also provide sufficient evidence. A single study in one species and sex might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites.

In the case of α-methylstyrene there are three neoplastic findings:

- Hepatocellular adenomas and carcinomas in female and male mice
- Renal tubular adenomas and carcinomas in male rats
- Mononuclear cell leukaemia in male rats

Increases in malignant tumours have been observed in two species, so Category 1B has to be considered.

A single study in one species and sex might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree. However, this is not the case for any of the three tumour types listed above. Further, the findings at multiple sites in one species and sex (here kidney tumours and leukaemia in male rat) cannot be regarded as 'strong'.

To aid in the weight of evidence assessment, the CLP Regulation further provides a list of factors increasing or decreasing the level of concern for human carcinogenicity (CLP, Annex I, 3.6.2.2.6). An overview of these factors together with relevant information on α-methylstyrene is provided in the following table.

Factors increasing or decreasing the level of concern for human carcinogenicity	
Factor	**Evidence for α-methylstyrene**
Tumour type and background incidence	All three tumour types occur in humans High background incidence of hepatocellular tumours in B6C3F1 mice and of mononuclear cell leukaemia in F344 rats
Multi-site responses	Yes, in male rats
Progression of lesions to malignancy	Yes
Reduced tumour latency	Slightly reduced latency of mononuclear cell leukaemia
Whether responses are in single or both sexes	Renal tumours and mononuclear cell leukaemia in a single sex (male rats) Hepatocellular tumours in mice: a marked increase in females, a weak increase in males
Whether responses are in a single species or several species	Response in two species Each tumour type in a single species
Structural similarity to a substance(s) for which there is a good evidence of carcinogenicity	Cumene classified as Carc. 1B, but besides similarities the carcinogenicity profiles of the substances show also significant differences (α-methylstyrene did not cause an increase in respiratory tract tumours)

Factors increasing or decreasing the level of concern for human carcinogenicity	
Factor	**Evidence for α-methylstyrene**
Routes of exposure	Inhalation is a route relevant for humans
Comparison of ADME between test animals and humans	Information is limited but no indication of marked interspecies differences
The possibility of a confounding effect of excessive toxicity at test doses	No excessive toxicity
Mode of action and its relevance for humans	Renal tumours in male rats partly related to α2μ-globulin accumulation and increased cell proliferation MoA of liver tumours and mononuclear cell leukaemia unknown

To sum up, the main factors increasing the concern are:

- Increases in malignant tumours in two species
- Multi-site response in male rats

The main factors decreasing the concern are:

- High spontaneous incidence of hepatocellular tumours in B6C3F1 mice and high susceptibility of this strain to induction of liver tumours by chemicals
- High spontaneous incidence of mononuclear cell leukaemia in F344 rats, not seen in other strains and species, and poor reproducibility observed for some substances
- Involvement of α2μ-globulin accumulation in the development of renal tumours in male rats (although human relevance cannot be completely excluded)
- The fact that kidney tumours and mononuclear cell leukaemia were limited to a single sex a species

Taking into account all the factors increasing and decreasing the concern, RAC concludes that α-methylstyrene should be classified as **Carc. 2; H351** as proposed by the DS.

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ANNEXES:

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).